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Supplemental Information

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Supplemental Figures and Legends

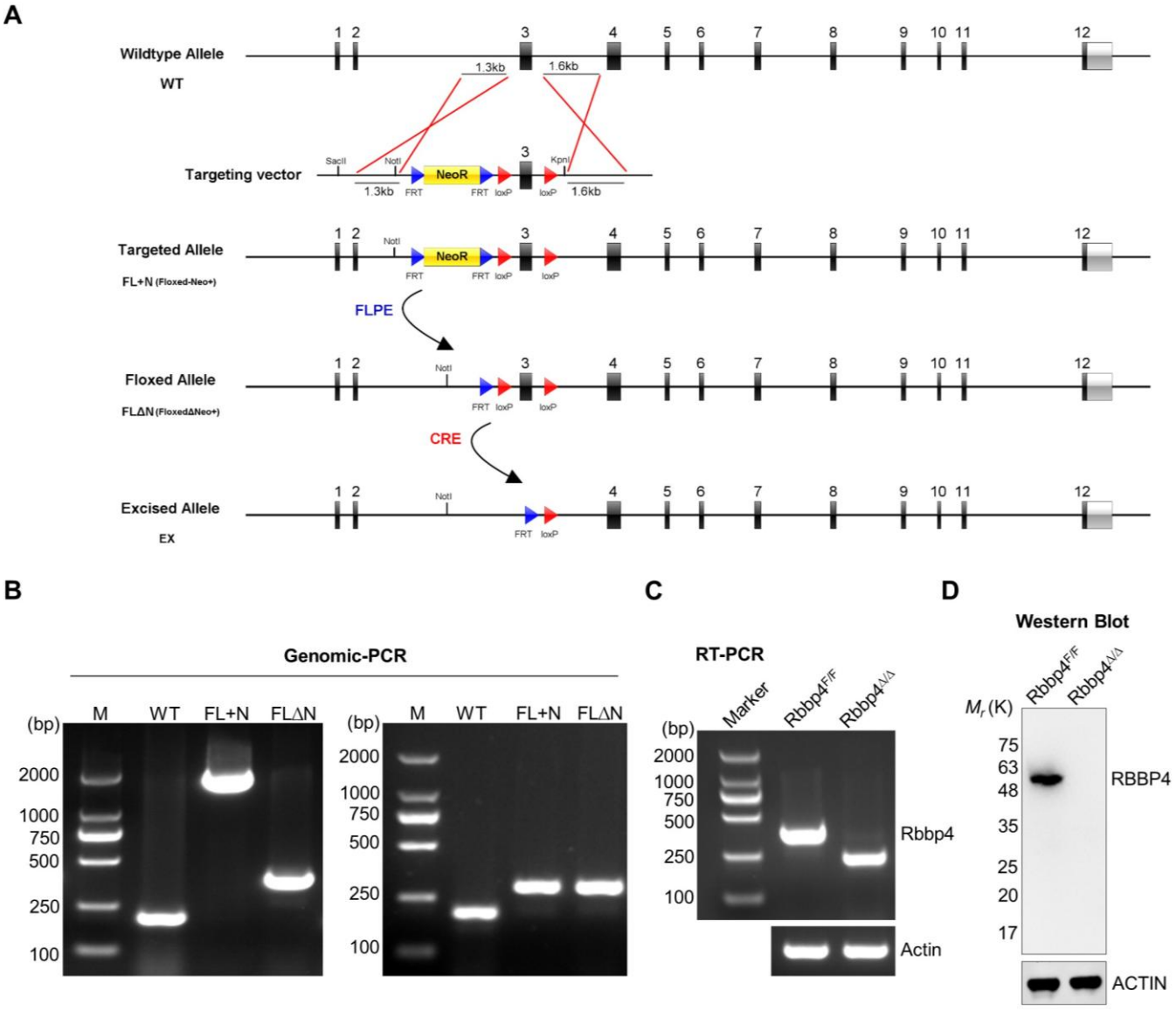

Figure S1. Generation of Conditional Inactivation of *Rbbp4* in ESCs, Related to Figure 1

(A) Overview of the gene targeting strategy. The wildtype *Rbbp4* locus, *Rbbp4* gene-targeting vector, the targeted allele and the excised allele are shown. (B) Genomic PCR analysis of the targeted allele and the floxed allele. (C) RT-PCR analysis for residual *Rbbp4* mRNA revealing a shorter band by transient expression of Cre recombinase. (D) Western blot analysis using a specific antibody against RBBP4 demonstrating absence of the protein in the sample three days after transient expression of Cre. ACTIN served as a loading control.

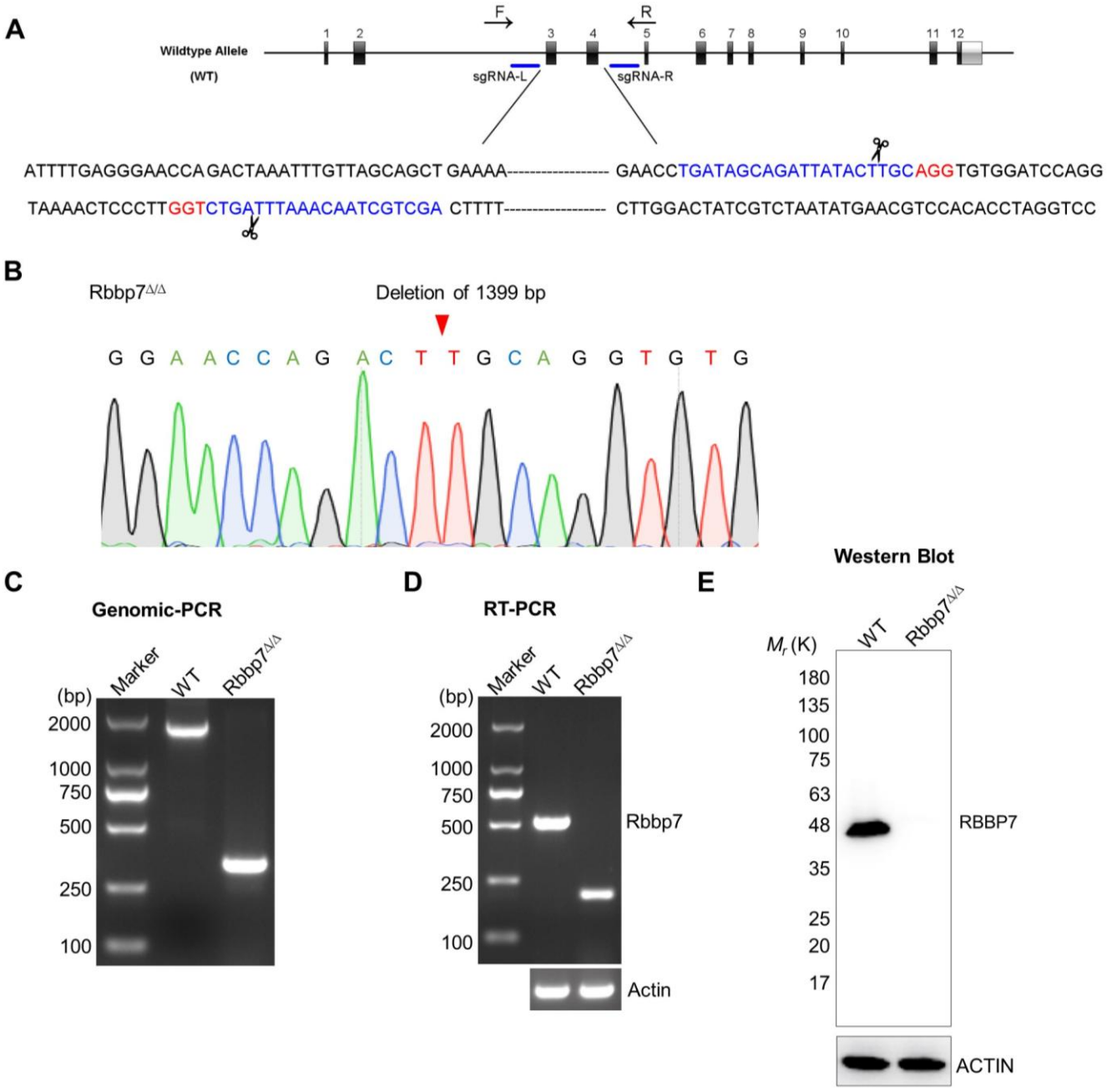

Figure S2. Generation of *Rbbp7* Null ESCs by CRISPR-Cas9, Related to Figure 1

(A) Schematic illustration for production of *Rbbp7* knockout ESCs via CRISPR/Cas9-mediated gene targeting. The sgRNA sequences are highlighted in blue and PAM sequences are in red. Primers used for PCR and sequencing are depicted as gray arrows. (B) Direct sequencing of the PCR product from *Rbbp7*$^{\Delta/\Delta}$ ESCs determining deletion of 1399bp DNA fragment. (C) Genotyping of *Rbbp7*$^{\Delta/\Delta}$ ESCs using primers on the both sides of targeted region. (D) RT-PCR analysis of *Rbbp7*$^{\Delta/\Delta}$ ESCs showing a shorter band in the mutant. *β-actin* was shown as a loading control. (E) Western blot analysis revealing the absence of RBBP7 protein in *Rbbp7*$^{\Delta/\Delta}$ ESCs. ACTIN was shown as a loading control.

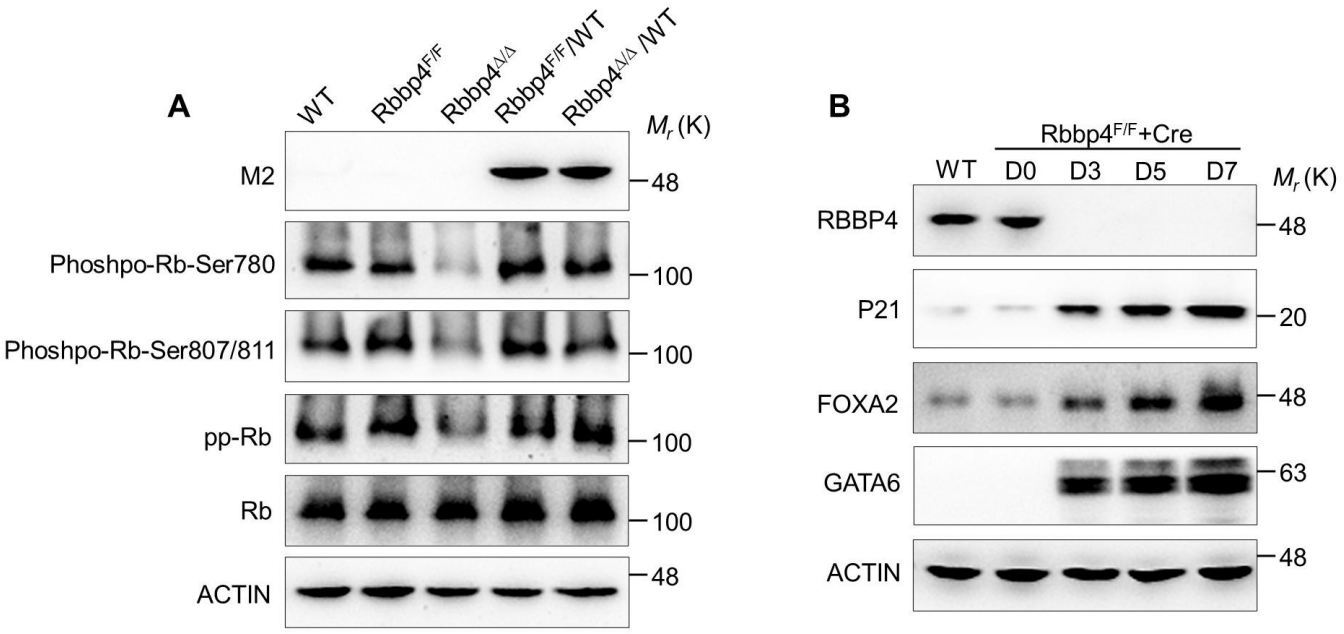

Figure S3. Loss of *Rbbp4* resulting in reduced levels of the phosphorylated and hyperphosphorylated forms of the Rb protein (ppRb), Related to Figures 1 and 2

(A) Western blot analysis of the phosphorylated and hyperphosphorylated forms of the Rb protein in ESCs of the indicated genotypes. (B) Western blot for P21, FOXA2 and GATA6 in *Rbbp4*$^{F/F}$ following transfection with Cre. ACTIN was used as a loading control.

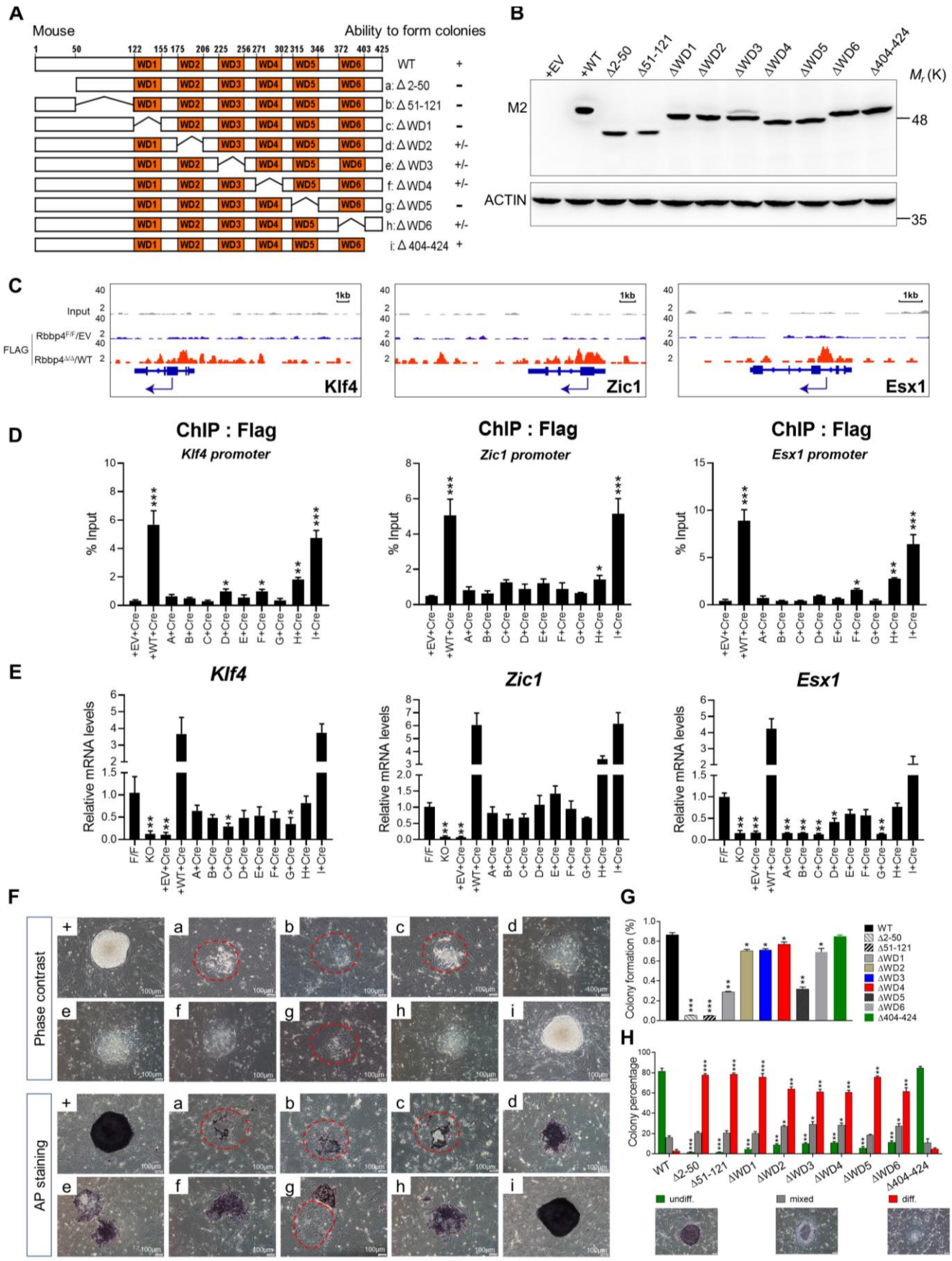
A
Mouse
Ability to form colonies
WT +
a: Δ 2-50 -
b: Δ 51-121 -
c: ΔWD1 -
d: ΔWD2 +/-
e: ΔWD3 +/-
f: ΔWD4 +/-
g: ΔWD5 -
h: ΔWD6 +/-
i: Δ 404-424 +
B
+EV
+WT
Δ2-50
Δ51-121
ΔWD1
ΔWD2
ΔWD3
ΔWD4
ΔWD5
ΔWD6
Δ404-424
Mr (K)
M2
ACTIN
48
35
C
Input
FLAG
Rbbp4F/F/EV
Rbbp4Δ/Δ/WT
1kb
Klf4
Zic1
Esx1
D
ChIP : Flag
Klf4 promoter
Zic1 promoter
Esx1 promoter
% Input
+EV+Cre
+WT+Cre
A+Cre
B+Cre
C+Cre
D+Cre
E+Cre
F+Cre
G+Cre
H+Cre
I+Cre
E
Klf4
Zic1
Esx1
Relative mRNA levels
F/F
KO
+EV+Cre
+WT+Cre
F
Phase contrast
AP staining
G
Colony formation (%)
WT
Δ2-50
Δ51-121
ΔWD1
ΔWD2
ΔWD3
ΔWD4
ΔWD5
ΔWD6
Δ404-424
H
Colony percentage
undiff.
mixed
diff.

Figure S4. Schematic domain structures and function analysis of Rbbp4 and its deletion mutants, Related to Figure 3

(A) Schematic domain structures and function analysis of RBBP4 and its deletion mutants. A concise schematic diagram of known structural characteristics of wild-type RBBP4 is shown at the top of the panel. The thin broken lines represent deleted regions. Results of pluripotency rescue assays using *Rbbp4*$^{F/F}$ ESCs reexpressing wild-type RBBP4 or deletion mutant proteins are presented on the right. +, rescue; +/–, partial rescue; –, no rescue. (B) The expression of different *Rbbp4* deletion mutant proteins in *Rbbp4* null ESCs. Protein levels of corresponding RBBP4 mutants were examined by western blotting with anti-FLAG antibody. ACTIN served as the loading control. (C) RBBP4 peaks at the indicated pluriptency-associated gene locus. (D) ChIP-qPCR analysis of RBBP4 binding to the select promoter regions in wild-type and deletion mutants. (E) Real-time PCR detecting *Klf4*, *Esx1*, and *Zic1* expression levels in indicated ESCs reexpressed wild-type and deletion mutants. Data were normalized to *β-actin* and values are represented as mean ± SD, (n=3). (F) Summary of results from rescue of *Rbbp4* deletion mutants. Morphology of respective mutant ESC colony with (bottom) or without (top) AP staining. The outlines of obviously defective colonies are circled. Scale bar, 100μm. (G) Percentage of isolated single ESCs of the indicated genotypes giving rise to macroscopic colonies. (H) Quantitative analysis of colony formation assay in ESCs of indicated genotypes. AP-stained colonies were scored as undifferentiated (undiff.), mixed or differentiated (diff.). Data are plotted as mean ± SD of three independent experiments in triplicates. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ (Student's t test) compared with the control.

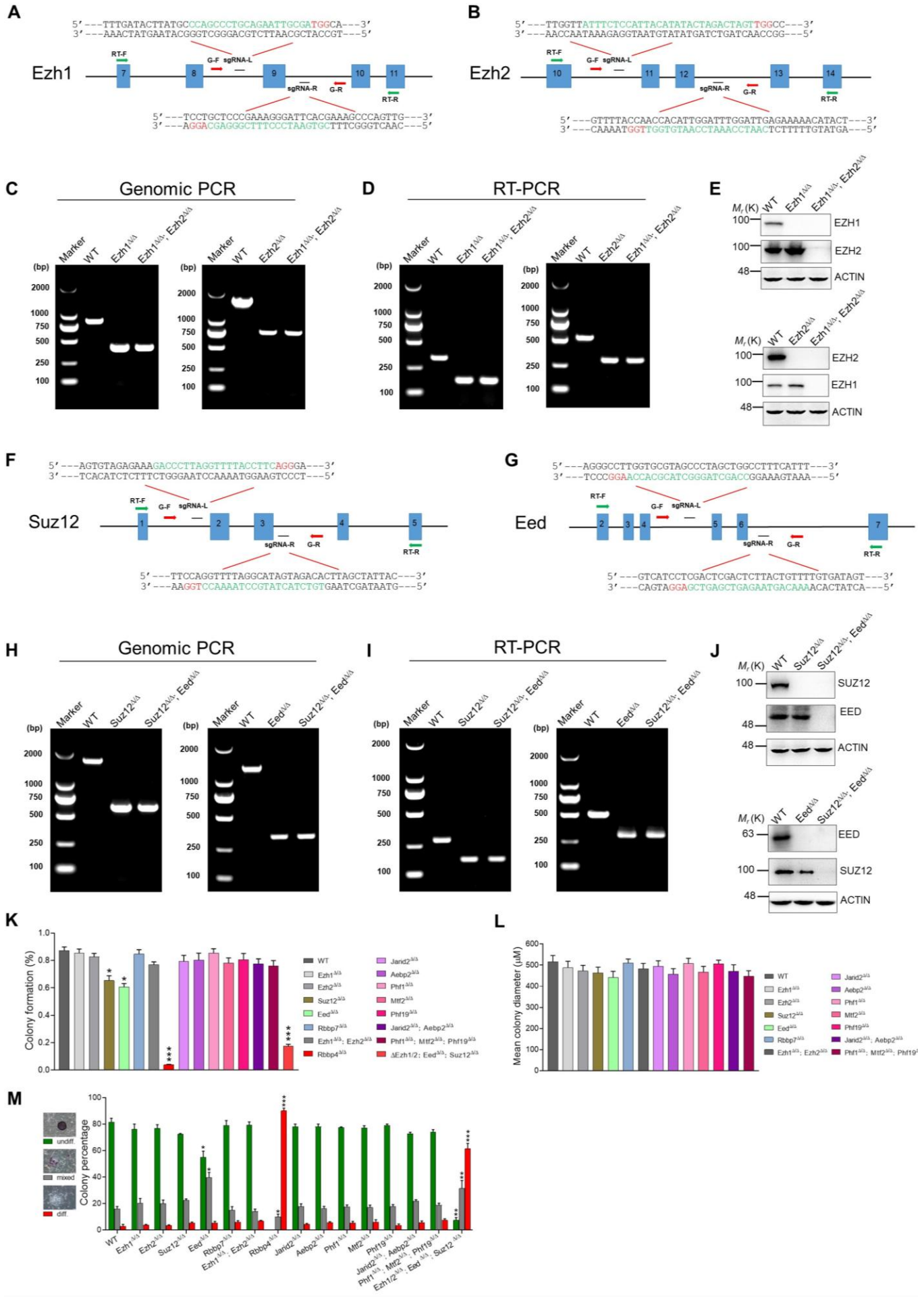
A
Ezh1
RT-F
G-F
sgRNA-L
sgRNA-R
G-R
RT-R
B
Ezh2
C
Genomic PCR
Marker
WT
Ezh1Δ/Δ
Ezh1Δ/Δ; Ezh2Δ/Δ
Ezh2Δ/Δ
(bp)
D
RT-PCR
E
Mr(K)
EZH1
EZH2
ACTIN
F
Suz12
G
Eed
H
Genomic PCR
Suz12Δ/Δ
Suz12Δ/Δ; EedΔ/Δ
EedΔ/Δ
I
RT-PCR
J
SUZ12
EED
K
Colony formation (%)
L
Mean colony diameter (μM)
M
Colony percentage
undiff.
mixed
diff.

Figure S5. Generation of Single Knockout and Combined Knockout ESC lines of PRC2 Core Subunits, Related to Figure 7

(A, B, F, G) Schematic overview of the strategy to generate *Ezh1*, *Ezh2*, *Suz12* and *Eed* knockout ESCs, respectively. PAM sequences are in red following the sgRNA sequence highlighted in green. The locations of genomic PCR primers (G-F, Forward; G-R, Reverse) are indicated by red arrows. (C, H) Genotyping of indicated knockout ESCs using primers upstream and downstream of the deleted region. (D, I) RT-PCR analysis for residual mRNAs revealing a shorter band in corresponding mutants. RT-PCR primers were represented by green arrows. *β-actin* served as a loading control. (E, J) Western blot analysis using specific antibodies recognizing related proteins demonstrating absence of the protein in knockout ESC lines. (K) Percentage of isolated single ESCs of the indicated genotypes giving rise to colonies. (L) Bar graphs summarize the mean diameter of 30 random ESC colonies of the indicated genotypes. (M) Quantitative analysis of colony formation assay in ESCs of indicated genotypes. AP-stained colonies were scored as undifferentiated (undiff.), mixed or differentiated (diff.). Data in (K) to (M) represent as mean ± SD obtained from three independent experiments, $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ (Student's t test) compared with the control.

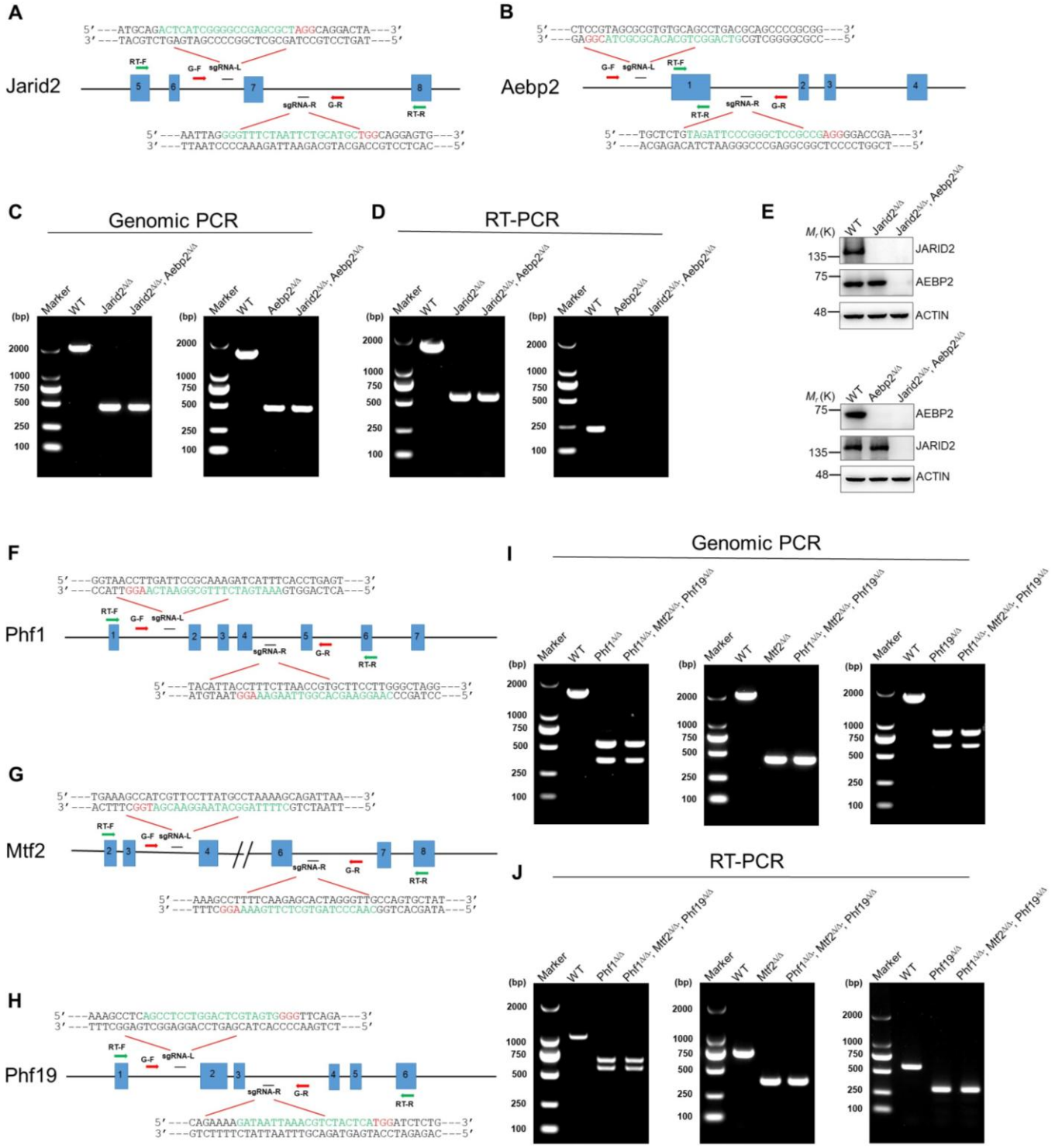

Figure S6. Generation of *Jarid2*, *Aebp2* or *Pcl1-3* Knockout ESCs, Related to Figure 7

(A, B, F, G, H) The schematic of *Jarid2*, *Aebp2* and *Pcl1-3* gene targeting strategies, respectively. Deleted regions are shown on the corresponding panels. The locations of genomic PCR primers (G-F, Forward; G-R, Reverse) are shown by red arrows, and RT-PCR primers (RT-F, Forward; RT-R, Reverse) are represented by green arrows. (C, I) Genomic PCR for analysis of indicated knockout ESCs. (D) RT-PCR analysis for residual *Jarid2*, *Aebp2* and *Pcl1-3* mRNA. (E) The absence of the proteins JARID2 or AEBP2 in knockout ESCs analyzed by Western blot. ACTIN served as a loading control.

Supplemental Tables

Table S1. All sgRNA sequences and primer sequences for genomic PCR and RT-PCR used in this study.

SgRNAs (related to Figures. 1, 7 and S1, S2, S5 and S6)		
Gene Name	**Upstream sgRNA**	**Downstream sgRNA**
Rbbp4	GTGATGACTAATTGTCCTCT	GGCCCAGGTTCAATTCCCAA
Rbbp7	AGATGGGAAGCAGGGTTTTG	TGATAGCAGATTATACTTGC
Ezh1	CCAGCCCTGCAGAATTGCGA	CGTGAATCCCTTTCGGGAGC
Ezh2	ATTACATATACTAGACTAGT	CAATCCAAATCCAATGTGGT
Suz12	GACCCTTAGGTTTTACCTTC	TGTCTACTATGCCTAAAACC
Eed	CCAGCTAGGGCTACGCACCA	AAACAGTAAGAGTCGAGTCG
Jarid2	ACTCATCGGGGCCGAGCGCT	GGGTTTCTAATTCTGCATGC
Aebp2	GTCAGGCTGCACACGCGCTA	TAGATTCCCGGGCTCCGCCG
Phf1	AAATGATCTTTGCGGAATCA	CAAGGAAGCACGGTTAAGAA
Mtf2	CTTTTAGGCATAAGGAACGA	CAACCCTAGTGCTCTTGAAA
Phf19	AGCCTCCTGGACTCGTAGTG	GATAATTAAACGTCTACTCA
Pou5f1	GGATTACATGCTCGTTCGTC	TACTGGCTTTCTCCAACCGC
Primers_Genomic PCR (related to Figures. S1, S2, S5 and S6)		
Gene Name	**5' Forward**	**5' Reverse**
Rbbp4	GGATCCCTCAGTCTCACCAG	TCCTGCCTCATGTGCCTTAG
Rbbp7	AGATGGGAAGCAGGGTTTTG	TCATTGTTGAAGCTGCTGGT
Ezh1	CTGCTTTTGAGGACGTCTGG	TTGACTGGAGTGAGCAGAGG
Ezh2	GTACCCTCTTCCCCTGTTCC	ACACAGTGGGTTGAGGTAGG
Suz12	TGGCGTAAATGTGTGTGTGG	CGCGCGTGTTCTCATCTTTA
Eed	AGGGTGACACTGAATCTGCA	CTCTCTCCTGCCACCTACAC
Jarid2	AGTGTGCTGGGTTCTTGCTT	AACCCTAAAACCCCAGGATG
Aebp2	CTTGCTCCACTTACGGCTTC	CTTGCTCCACTTACGGCTTC
Phf1	GGGGCACTCAGGGAAGTTAT	TGCAGAGGGACAGACAGATG
Mtf2	AGTGACGCTGGGTCTTGTCT	ACACTCCAAAGTGGCAAAGG
Phf19	GGGGAGAGCCAGATGTGTTA	ATGGCCACTTTCATGCTTTC
Primers_RT-PCR (related to Figures. S1, S2, S5 and S6)		
Gene Name	**5' Forward**	**5' Reverse**
Rbbp4	GGAAGAACGGGTGATCAACG	AGGGGTTCTGAGGCATGTAC
Rbbp7	TTCAGTGGCCCAGTCTTACC	TTTGCCTTCCTTTGGTCCTG
Ezh1	CCATTGCGTCCATGTTTCCT	CATGGCGTACTCCTTTGCTC
Ezh2	AAAGACACCACCTAAACGCC	ACATGGTTAGAGGAGCCGTC
Suz12	AGAAGCCGAAAATGGAGCAC	TGTGGAAGAAACCGGTAAATGT
Eed	AGGAAGGAAAAGCTGGGGAA	CCCTTCCACACCTCCGAATA
Jarid2	CTTCATCTTCGTGCCAGTCA	CAGGGAGTGGAACTTGTGGT
Aebp2	GACGAGGACGAGGAGGAC	CACGCTACTGCTGCTGCTAC
Phf1	CCTTCTGTCGACTCCTCCTG	TTACAGCAAACGCTGAGGTG
Mtf2	CAAGCGGTCTCCTTTACGTC	TGGTCCAGAACTGCAGACAG
Phf19	CAAGTGGGACCGGCTGAC	GCTGGTAGGACAGCACCATT

Table S2. All primer sequences for RT-qPCR and ChIP-qPCR used in this study.

Primers_RT-qPCR (related to Figures. 2, 4, 5, 6 and 7)		
Gene Name	**5' Forward**	**5' Reverse**
Dppa5a	CAGGCCATGTTTGAGCTGAA	ACTCGATACACTGGCCTAGC
Fgf4	TCTGCCCAACAACTACAACG	GAGGGGTAGGGTGTGCTTC
Lefty1	CGCACTGCCCTTATCGATTC	ACTTGTGTGAGCTCCAGGTT
Nanog	ATGCGGACTGTGTTCTCTCA	CCGCTTGCACTTCATCCTTT
Utf1	AGACTCTGCCTACTTACCGC	TCTGGTTATCGAAGGGTCCG
Foxd3	CGAGTTCATCAGCAACCGTT	GGTCCAGGGTCCAGTAGTTG
Tcl1	TTTCCTCTACACTGGGCTCC	GAGGAGGTGAGGAGAACTGG
Sox2	CGCGGAGTGGAAACTTTTGT	CGGGAAGCGTGTACTTATCC
Pou5f1	GGATGGCATACTGTGGACCT	TCTCCAACTTCACGGCATTG
Nr5a2	TGAAGCAGCAGAAGAAAGCC	TGTCATAGTCTGTCGGAGGC
Sox17	GCCGAGCCAAAGCGG	GTCAACGCCTTCCAAGACTTG
Fgf5	TTGCGACCCAGGAGCTTAAT	CTACGCCTCTTTATTGCAGC
Brachyury	CCAAGGACAGAGAGACGGCT	AGTAGGCATGTTCCAAGGGC
Flk1	GCTTGCTCCTTCCTCATCTC	CCATCAGGAAGCCACAAAGC
Gata6	CCCACTTCTGTGTTCCCAATTG	TTGGTCACGTGGTACAGGCG
Foxa2	CCCTACGCCAACATGAACTCG	GTTCTGCCGGTAGAAAGGGA
Sox11	AGAACATCACCAAGCAGCAG	TCCTTATCCACCAGCGACAG
Nestin	AGGTGTCAAGGTCCAGGATG	AAGGAAGCAGACTCAGACCC
Gata4	AAACCAGAAAACGGAAGCCC	ATAGTGAGATGACAGCCCGG
Msx2	CCTATCAACTCACCCCTGCA	CATTCAGGAGCAGAGTTGGC
Hand1	CAGCTACGCACATCATCACC	GAAATCTGGGGCAGCATCAG
Gata2	GATGAATGGACAGAACCGGC	TTCTTCATGGTCAGTGGCCT
Ets2	GGCACCAAACTACCCCAAAG	GTCGTGGTCCTTGGGTTTTC
Cdx2	GAAACCTGTGCGAGTGGATG	CAGCCAGCTCACTTTTCCTC
Cyp11a1	CGAGTTCACAGGCTGCATAC	ACTCCGCTAACCACACAGAA
Gata3	CCCTTCTCCAAGACGTCCAT	CTTTCTCATCTTGCCTGGCC
Actin	AGCCATGTACGTAGCCATCC	CTCTCAGCTGTGGTGGTGAA
Hprt1	GAATCCTCTGGGAGACGACA	CGGAAAGCAGTGAGGTAAGC
Gadd45g	AAGCACTGCACGAACTTCTG	ACGTGAAATGGATCTGCAGC
Plk2	CTCCGGACAGACTCTCTTCC	CTAGGCTGCTGGGTTATCGA
Creb3	TGTCACCTTCCGAGAACTCC	GAACCCCTCCTTTTCGAAGC
Ccnb1	TTGTGTGCCCAAGAAGATGC	ACGTCAACCTCTCCGACTTT
Cdkn1a	GAGAAAACCCTGAAGTGCCC	GTTTGGAGACTGGGAGAGGG
Klf4	GACATCAATGACGTGAGCCC	TGGGCTTCCTTTGCTAACAC
Esx1	AAACTACCAGGAACCCGAGG	ATCAGAGGACGCATCAGGG
Zic1	TGAACATGGCTGCACATCAC	AGATGTGGTTGCTCTGCTCT
Cdkn2b	CTGCCACCCTTACCAGACC	GCAGATACCTCGCAATGTCA
Ccna1	ACAGACCCAAGGCTCACTAC	TCCAGGAAGTTGACAGCCAA
Hmga2	CAAGAGCCAACCTGTGAGC	ACGACTTGTTGTGGCCATTT

Ccnd1	ACTGACAACTCTATCCGGCC	CGGATGGTCTGCTTGTTCTC
Cdc6	TCCTCCTCCGCTCAAAGAAG	ATCCGGGACAGCTGTATTCA
Krt18	CTGGGGCCACTACTTCAAGA	ATCTACCACCTTGCGGAGTC
Tbx2	GCCTGGACAAGAAAGCCAAA	TGGTCAGCTTCAGTTTGTGG
Bmp2	CTGGGGCCACTACTTCAAGA	ATCTACCACCTTGCGGAGTC
Sox7	GAACACGCTGCCTGAGAAAA	AGGACGAGAAGAAGGTCTGC
Pax6	TCTGCAGGTATCCAACGGTT	GCAAAGATGGAAGGGCACTC
Ncam1	GAATCAGACGGCCATGGAAC	TGCTCTTCAGGGTCAAGGAG
Hesx1	TACATGCCCCAGATCTTCCC	TACTTCGACCTGGTTCTGGG
Neurod1	ATCAAAAGCCCAAGAGACGG	GGGTCTTGGAGTAGCAAGGT
Olig1	GCAGCCACCTATCTCCTCAT	CAAGTTCAGGTCCTGCATGC
Olig2	CTGCGCCTGAAGATCAACAG	CGTAGATCTCGCTCACCAGT
Esrrb	TCTCATCTTGGGCATCGTGT	AGTTTCTTGTACCTGCGCAC
Zfp296	ATTAGGGGCCATCATCGCTT	GCACAGCAACTTCCAAGGAC
Nr0b1	CTATGTGTGCGGTGAAGAGC	CCTTGAGTGTGATCAGACGC
Gbx2	TCCCGGCCATTTCGTCTAC	CTGGGGATCTGGTGGTGAG
Lefty2	CATCGACTCTAGGCTCGTGT	CGAAACGGACCAACTTGTGT
Primers_ChIP-qPCR (related to Figures. 3, 7 and S4)		
Gene Name	**5' Forward**	**5' Reverse**
Zic1	CCTTCAAGCTCAACCCCAGT	GGAACAGAAAGTCCCGGGTA
Esx1	CTAGAGTGAGGCCCTTACCC	AGAGAGTCTGGGAAAGGGGA
Klf4	GAAGGGAGAAGACACTGCGT	GCACTTAAGGCCGACTCAAG
Brachyury	GGGTCGCTATCTGTTCGTCT	TGACCTCTCCAAAGCCTCAG
Hand1	CAAGATCAAGACTCTGCGCC	TTCTTACTTACCCCAGCCCG
Sox17	ATACGCCAGTGACGACCAG	GTTCGTCTTTGGCCCACAC
Gata4	GGCCCGTGTAATCTCTCTGA	CGCAAGTTTCCGAGCCTATC
Cdx2	ACCATCACCCGCATCATCA	CATCCACTCGCACAGGTTTC
Tbx2	CCTTACGGCGGGTCAGATC	CAGGGAGAAGGTGTCGGAAG
Hprt1	GAATCCTCTGGGAGACGACA	CGGAAAGCAGTGAGGTAAGC
Pou5f1	CAGACCCTTGTAGTGCCTGA	AGGCCATCAGACACTAAGCA
Sox2	AGCTCGCAGACCTACATGAA	TGGAGTGGGAGGAAGAGGTA
Nanog	CGGCTCACTTCCTTCTGACT	CATGTCAGTGTGATGGCGAG

Table S3. Antibodies used in this study, related to Figures 1, 5, 7 and S1-S6.

Antibodies	**SOURCE**	**IDENTIFIER**
Rabbit anti-Rbbp4	Bethyl	A301-206A-A
Rabbit anti-Oct4	Cell Signaling Technology	83932S
Mouse anti-Sox2	Cell Signaling Technology	4900s
Mouse anti-Nanog	Abcam	ab214549
Rabbit anti-Rbbp7	Cell Signaling Technology	#4522
Mouse anti-Ezh1	Santa Cruz Biotechnology	sc-398767
Rabbit anti-Ezh2	Cell Signaling Technology	5246S
Goat anti-Suz12	Santa Cruz Biotechnology	sc-46264
Sheep anti-Eed	R&D systems	AF5827
Rabbit anti-Jarid2	Cell Signaling Technology	13594S
Rabbit anti-Aebp2	Cell Signaling Technology	14129S
Rabbit anti-Foxa2	Abcam	ab108422
Rabbit anti-Gata6	Cell Signaling Technology	5851S
Rabbit anti-Cdx2	Cell Signaling Technology	12306S
Rabbit anti-Flag	Sigma-Aldrich	F1804
Rabbit anti-HA	Santa Cruz Biotechnology	sc-805
Rabbit anti-Ring1b	Proteintech	16031-1-AP
Rabbit anti-H3K27me3	Cell Signaling Technology	9733S
Rabbit anti-H2AK119ub1	Cell Signaling Technology	8240S
Rabbit anti-H3	Cell Signaling Technology	14269S
Rabbit anti-phospho-RB(Ser780)	Cell Signaling Technology	8180t
Rabbit anti-phospho-RB(Ser807/811)	Cell Signaling Technology	8516T
Rabbit anti-RB	Abcam	ab181616
Rabbit anti-p21	Cell Signaling Technology	#64016
Mouse anti-ppRB	BD Pharmingen	G3-245
Mouse anti-Actin	Proteintech	60008-1-Ig
Alex594-conjugated donkey anti-mouse IgG	Cell Signaling Technology	8809s
Alex488-conjugated donkey anti-rabbit IgG	Cell Signaling Technology	4412s
Alex488-conjugated donkey anti-mouse IgG	Cell Signaling Technology	4408s
Alex594-conjugated donkey anti-rabbit IgG	Cell Signaling Technology	8889s

Table S4. Additional datasets used in this study, related to Figures 2 and 3.

RNA-Seq Datasets		
Gene Name	**SOURCE**	**IDENTIFIER**
Suz12	(Pasini et al., 2007)	GSE31354
Ezh2	(Das et al., 2015)	GSE58414
Eed	(Das et al., 2015)	GSE58414
Oct4	(Loh et al., 2006)	GSE4189
Sox2	(Ding et al., 2015)	GSE66736
Nanog	(Loh et al., 2006)	GSE4189
ChIP-Seq Datasets		

Gene Name	SOURCE
Suz12	(Ben-Porath et al., 2008)
Eed	(Ben-Porath et al., 2008)
H3K27me3	(Ben-Porath et al., 2008)

Supplemental Experimental Procedures

Cell Culture

Mouse Embryonic stem cells (mESCs) were grown on 0.1% gelatin-coated culture dishes in DMEM medium supplemented with 15% fetal calf serum (Gibco) (v/v), 1,000 U/ml leukaemia inhibitory factor (LIF), 100 U/ml penicillin, 100 U/ml streptomycin (Thermo), 2 mM L-glutamine (Invitrogen), 1:100 non-essential amino acids (Invitrogen) and 0.1 mM β-mercaptoethanol (Sigma-Aldrich). HEK293FT and mouse embryonic fibroblasts (MEFs) were cultured in DMEM media containing 10% fetal calf serum (Gibco) (v/v), 100 U/ml penicillin-streptomycin (Thermo). MEFs were derived from E13.5 embryos and cultured as previously described (Qin et al., 2010). All cell lines used in this study were maintained in a humidified incubator at 37 ℃ and 5% CO2.

Colony Formation, Alkaline Phosphatase Staining and Flowcytometry

For colony formation assay, trypsinized ESCs were seeded on mitomycin-treated feeder MEFs (about 1,000 cells per 10cm plate) and grown for 7 days before image acquisition by phase contrast microscope (Olympus TH4-200). ESC colonies were stained with Alkaline Phosphatase Detection Kit (Yeasen #40749ES60), according to the manufacturer's guidelines. After staining to reveal AP activity, the colonies were scored and the percentage of undifferentiated, mixed and differentiated colonies was calculated. (n=3; error bars indicate the mean ± s.d.). Cell cycle analysis was performed, as previously described (Zhao et al., 2018). The experiment was carried out in strict accordance with the manufacturer's instructions (Vazyme A411-01/02). In short, 2–5 ×10^6 ES cells were trypsinised, washed in PBS, and then fixed dropwise into ice-cold 75% ethanol overnight at 4 ℃. Whereafter, fixed cells were washed with PBS, incubated for 30 min at 37°C in precooling PBS containing 20μl RNase A Solution and stained with propidium iodide (PI) at 4 ℃ in dark for 30 min. For apoptosis analysis, Annexin V-FITC/PI apoptosis detection kit (Yeasen #40302ES60) was used. Briefly, 2–5 ×10^5 cells were harvested, washed and resuspended in 100μL binding buffer containing 5μl Annexin V-FITC and 10μl PI at room temperature in the dark for 10 min. Fluorescence intensities were acquired using a LSRFortessa flow cytometer (BD Biosciences). The data were analyzed by the ModFit LT software and FlowJo software, respectively.

Embryoid Body Formation Assay

For Embryoid body differentiation, the protocol, hanging drop assay in Qin et al. was used (Qin et al., 2012). In short, a proper number of ESCs were seeded to non-adherent Petri-dishes. Suspended drop ESCs were maintained in IMDM medium without LIF (15% fetal calf serum, 2 mM L-Glutamine, 100 U/ml Penicillin-Streptomycin, non-essential amino acids, 50 μg/mL Ascorbic acid, 200 μg/mL iron saturated holo-transferrin, sodium pyruvate, 450 μM monothioglycerol, and 0.1 mM β-mercaptoethanol) to generate embryoid bodies (EBs). Three days later, the EBs were maintained on a rotating shaker using suspension culture method. The medium was replaced every 2 days throughout the differentiation

procedure. Pictures of EB morphology at day 6 and 12 were taken by Olympus (TH4-200). Total RNA was extracted (Trizol, Invitrogen) at the indicated time points and analyzed by RT-qPCR. All RT-qPCR primer sequences are available in Table S2.

Teratoma Formation Assay

5 x 10^6 ES cells were trypsinized, washed with PBS and injected subcutaneously into the flank of 6-week-old nude mice (the Model Animal Research Center of Nanjing University [D000521] BALB/c-*Foxn1*^{nu}/Nju). Teratomas were surgically isolated from mice 4 weeks after injection for routine histological analysis. Firstly, for preparation of teratoma paraffin blocks, teratomas were transferred into 4% paraformaldehyde (PFA) overnight at 4℃. Then, tissue samples were dehydrated and embedded in paraffin. Finally, 5-μm-thick paraffin sections were deparaffinized and stained with hematoxylin and eosin (H&E) based on a standard protocol (Qin et al., 2012; Zhao et al., 2018). All paraffin-sections subjected to histological examination after H&E staining. Images were acquired with a microscope (Olympus DP73). All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Model Animal Research Center at Nanjing University.

Cloning and Plasmid Generation

For construction of expression vectors for *Rbbp4* with an N-terminal Flag tag, the full-length *Rbbp4* coding sequence was amplified by PCR using Phanta Max Super-Fidelity DNA Polymerase (Vazyme #P505-d1) with primers containing the Flag tag sequence (DYKDDDDK). Mutant *Rbbp4* constructs were generated by Quick-change II site-directed mutagenesis kit (Agilent). The PCR product was inserted into lentiviral vector as described previously (Qin et al., 2012). In the same way, Full-length ORFs of *Oct4*, *Sox2* and *Nanog* were amplified with primers containing C-terminal Flag-tag or HA-tag from cDNA generated from ESCs. All ORFs were subsequently inserted between the NotI and BamHI sites of lentiviral vectors by recombination using ClonExpress II One Step Cloning Kit (Vazyme #C112-01), in accordance with manufacturer's instructions. For generation of *Rbbp4* targeting vector, an ~4.3kb NotI-KpnI fragment containing ~1.3 kb upstream and ~1.6 kb downstream homology arms, FRT flanked Neo cassette, floxed exon 3 and the neomycin positive selection marker was inserted into pBS KS II vector. And all expression vectors and targeting vector were verified by sequencing to ensure no mutations were introduced in protein coding sequence.

Lentiviral Supernatant Production and Infection

Lentiviral production and delivery were performed as previously described (Qin et al., 2012). Lentiviral particles were generated by co-transfecting HEK293FT cells with the lentiviral expression vectors along with third-generation packaging plasmids using Lipofectamine 2000 (Life Technologies). Concentrated virus particles were used to infect target cells in the presence of polybrene (Sigma, final concentration of 8 μg/ml). 24 hours after infection, ES cells were selected with puromycin (4 μg/ml) for 3–5 days to generate stable cell lines.

Immunofluorescence

For immunofluorescence, ESCs were plated on glass slides pre-coated with poly-L-lysine solution (Sigma-Aldrich). Standard immunofluorescence staining protocols were followed. Briefly, ESCs were fixed in 4%(w/v) paraformaldehyde for 15 min at room temperature, washed with PBS and then permeabilised in 0.5% Triton X-100 in PBS for 20 min at room temperature. After three 3 min-washes

in PBS, cells were incubated for 30 min in blocking buffer (PBS, 10% (v/v) goat serum, 1% (w/v) BSA) to block nonspecific binding sites. Next, the primary antibodies were diluted to the appropriate concentration in blocking serum at 4℃ overnight. Primary antibodies used were the following: anti-SOX2 (1/200, #4900s, Cell Signaling Technology), anti-OCT4 (1/200, sc-5279, Santa Cruz Biotechnology), anti-FOXA2 (1/200, ab108422, abcam), anti-GATA6 (1/200, 5851S, Cell Signaling Technology), and anti-CDX2 (1/200, 12306S, Cell Signaling Technology). After three 3 min-washes with PBS, cells were incubated for 1h at room temperature in the dark with the secondary antibody conjugated to fluorophores (Alexa Fluor® 488 donkey Anti-Mouse IgG (H+L) Antibody, Alexa Fluor® 488 donkey Anti-Rabbit IgG (H+L) Antibody, Alexa Fluor® 594 donkey Anti-Mouse IgG (H+L) Antibody and Alexa Fluor® 594 donkey Anti-Rabbit IgG (H+L) Antibody, Cell Signaling Technology) diluted 1:1000 in PBS 1% (w/v) BSA. Subsequently, coverslips were counterstained with DAPI (4′,6-diamino-2-phenylindole) (1:2000) for 5 min for nuclear detection at room temperature in the dark. After several more washes, cells were imaged with Zeiss LSM 880 laser scanning confocal microscope at 63X magnification.

Quantitative Real-time PCR

Total RNA was extracted (Trizol, Invitrogen) from cells or EBs and cDNA was generated by reverse transcription PCR using HiScriptTM 1st Strand cDNA Synthesis Kit (Vazyme #R111-01) according to the manufacturer's instructions. Relative mRNA expression levels were determined by PCR using PowerUp™ SYBR® Green Master Mix (Invitrogen) on the StepOnePlus™ Real–Time PCR System (Applied Biosystems). Data were analyzed with the ddCt method, using *β-actin* as a normalizer. Detailed primer sequences for RT-PCR are provided in the Table S1.

Histone Extraction

Histone extraction was performed as previously described (Qin et al., 2012; Zhao et al., 2017). In brief, the cells were harvested, washed and resuspended in Triton Extraction buffer (TEB) (PBS containing 0.5% Triton X 100 (v/v), 2mM phenylmethylsulfonyl fluoride, 0.02% (w/v) NaN3). Then the cells were shaken on ice for 10 min with gentle stirring and centrifuged at 6500 g for 10 min at 4℃ to spin down the nuclei. Supernatant was discarded and the nuclei pellet was washed in TEB and resuspended in 0.2 N HCL overnight at 4℃. Then the nucleic solution was centrifuged and supernatant was collected, and neutralized HCL with 5M NaOH at 1/25 of the volume of the supernatant. Protein content was determined using the Bradford assay.

Western Blotting and Immunoprecipitations

Total protein lysates were obtained by lysing cells in RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 5 mM EDTA, and protease inhibitors) as described (Huang et al., 2018). Cell lysates were separated by SDS-PAGE and transferred to PVDF membranes. For immunoprecipitation assay, immunoprecipitations were performed as follows. Cells were harvested and resuspended in 1ml of IP lysis buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1% TtitonX-100, 2 mM EDTA, Complete protease inhibitors). After a 30 min incubation on ice, anti-RBBP4 (A301-206A-A, Bethyl), anti-SUZ12 (sc-46264, Santa Cruz Biotechnology) and ANTI-FLAG® M2 Affinity Gel beads (A2220, Sigma) were added to lysates for immunoprecipitation. The lysates were incubated with rotation at 20 rpm at 4℃ overnight and centrifuged at 2000g for 5 min at 4℃. Beads were then washed two times with IP lysis buffer and high-salt buffer (50 mM Tris–HCl pH 8.0, 450 mM NaCl, 1% TtitonX-100, 2 mM EDTA, protease inhibitors),

respectively, and resuspended in 50 μl of SDS sample buffer (125 mM Tris–HCl pH 6.8, 2% SDS, 10% glycerol, 1 mg/ml bromophenol blue, 100 mM DTT, 2% β-mercaptoethanol) and boiled at 95 ℃ for 5 min. Samples were loaded on SDS-PAGE for western blot analysis. Detailed antibodies used in this study are provided in the Table S3.

RNA Sample Preparation and RNA-Seq

Total RNA was extracted from ESCs using TRIzol® Reagent according the manufacturer's instructions (Invitrogen) and genomic DNA was removed using DNase I (TaKara). Then high-quality RNA sample was quantified using the ND-2000 (NanoDrop Technologies). RNA purification, reverse transcription, library construction and sequencing were performed at Shanghai Majorbio Bio-pharm Biotechnology Co., Ltd. (Shanghai, China), as per the manufacturer's instructions (Illumina, San Diego, CA). Shortly, 1μg of total RNA was prepared following TruSeq™ RNA sample preparation Kit from Illumina (San Diego, CA) for RNA-seq transcriptome librariy construction. Then the library was subsequently sequenced with the Illumina Novaseq 6000. The raw sequencing data were aligned to mouse genome (Ensemble GRCm38.p5) with orientation mode using TopHat (version2.1.1) (Langmead and Salzberg, 2012). To identify differentially expressed genes (DEGs), the expression level of each transcript was calculated according to the fragments per kilobase of exon per million mapped reads (FPKM) method. RSEM (http://deweylab.biostat.wisc.edu/rsem/) (Li and Dewey, 2011) was used to quantify gene abundances. R statistical package software EdgeR (Empirical analysis of Digital Gene Expression in R) (Robinson et al., 2010) was utilized for differential expression analysis. RNA-seq data in this study were deposited to the Gene Expression Omnibus (accession number. GSE144155).

Chromatin Immunoprecipitation (ChIP)

ChIP was performed as described previously (Qin et al., 2012; Zhao et al., 2017). In brief, 10^7 ES cells were collected and cross-linked for 10 min at room temperature with 1% formaldehyde (Sigma). The cross-linking reaction was terminated by the addition of glycine (0.125 M) for 5 min at room temperature followed by cell lysis (50 mM Tris pH 8.0, 1% SDS, 10 mM EDTA, protease inhibitors) at 4˚C for 20 min. Sonication was performed on a Bioruptor (Diagenode), resulting in an average DNA fragment size of 200-500 bp. Before immunoprecipitation, a 50 μl aliquot of solubilized chromatin was transferred to a new microtube and stored at −20˚C as 'input'. The fragmented chromatin fragments were incubated overnight in dilution buffer (16.7 mM Tris pH 8.0, 0.01% SDS, 1.2 mM EDTA, 1.1% Triton X-100, 165mM NaCl, protease inhibitors) at 4 ℃ under rotation with the indicated antibodies ((anti-EZH2, 5246S, Cell Signaling Technology)(anti-SUZ12, ab12073, Abcam)(anti-EED, AF5827, R&D systems)(anti-RBBP4, A301-206A-A, Bethyl)(anti-H3K27me3, 9733S, Cell Signaling Technology)). After incubation, the beads coupled with immunocomplexes were then washed twice in low-salt buffer (twice) (150 mM NaCl, 20 mM Tris pH 8.0, 0.1% SDS, 1 mM EDTA, protease inhibitors), high-salt buffer (twice) (500 mM NaCl, 20 mM Tris pH 8.0, 0.1% SDS, 1 mM EDTA, protease inhibitors) and TE buffer (twice) (10 mM Tris pH 8.0, 0.25 mM EDTA, protease inhibitors). The immunocomplexes were eluted for 1h at 65˚C with 150 μl of elution buffer (1% SDS and 100 mM NaHCO3). Chromatin was decross-linked overnight in 0.3 M Nacl at 65˚C. The enriched DNA was treated with RNase A (0.2mg/ml) for 2 hr at 37 ℃, then Proteinase K (0.2mg/ml) for 3 h at 55 ℃. DNAs were then purified using DNA gel extraction kit (Axygen) for subsequent quantitative PCR or deep sequencing analyses. ChIP enrichment was analyzed by qPCR using specific primers for target genes and the data were normalized to input.

ChIP-Seq and Data Analysis

For ChIP-seq samples, cross-linking, sonication and IP were performed in *Rbbp4*$^{\Delta/\Delta}$ ESCs expressing RBBP4-Flag (*Rbbp4*$^{\Delta/\Delta}$*/WT*) or *Rbbp4*$^{F/F}$ ESCs expressing empty Flag vector (*Rbbp4*$^{\Delta/\Delta}$ */EV*) (control). Flag M2 antibody (1:1,000, catalogue number F1804, Sigma) was used in the ChIP assays. 5ng of qualified ChIP DNA were used to generate the sequencing library using a NEB kit and sequenced on the Illumina platform (HiSeq PE150). Quality filtered ChIP-seq reads were aligned to the reference genome (GRCm38/mm10) using the bowtie2 software package (version 2.1.0). Only uniquely and non-duplicate mapped reads were kept, then the reads coverage and depth were calculated by samtools. MACS2 was used to generate signal tracks files in BigWig format and normalized it to 1 million reads. The significantly enriched ChIP-seq peaks were identified by using MACS package with a P-value cutoff of 5×10^{-4}. Visualization of the mapped reads and peak calling was performed by bigwig files using IGV tools. MACS2 (version 2.1.1) was used to call peaks, and followed by peak annotation using bedtools. Differential analysis between treat and control samples was conducted using bedtools. ChIP-seq data presented in this study are accessible through GEO series accession number GEO: GSE155029.

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